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Characterization and *in vivo* efficacy of a heptapeptide ODT formulation for the treatment of neurogenic bladder dysfunction

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Abstract

The objective of this study was to develop oral disintegrating tablet (ODT) formulations of a heptapeptide, [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10), for the treatment of neurogenic bladder dysfunction. A design of experiment approach was applied to determine the optimal ratio of chosen excipients: gelatin (X₁), glycine (X₂), and sorbitol (X₃). These formulations were optimized for efficacy studies to produce ODTs exhibiting rapid disintegration times (Y₁) and appropriate structural integrity (Y₂) using JMP[®] 12.0.1 software. Based on theoretically predicted values from 12 experimental runs, the optimal ODT formulation was determined to be 3% (w/v) gelatin, 2% (w/v) glycine, and 1% (w/v) sorbitol in deionized water. Using this formulation, blank and drugloaded ODTs containing 1.5 mg or 5 mg of [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10) were manufactured by a lyophilization process. The peptide-loaded tablets disintegrated in less than 30 sec and released 97% of the peptide within 15 minutes. The peptide was stable for 90 days under 25 °C/60% relative humidity (RH) and 40 °C/75% RH. *In vivo* efficacy of the peptide-loaded ODTs was confirmed in a rat acute spinal cord injury model under isovolumetric bladder pressure recording conditions, concluding that sublingual administration of peptide-containing ODTs evoke a rapid dose-related neurokinin 2-mediated increase in bladder pressure.

GRAPHICAL ABSTRACT

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Orally disintegrating tablets containing a heptapeptide, [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10), prepared by a lyophilization method for the treatment of neurogenic bladder dysfunction. The peptide-loaded tablets disintegrated within 30 seconds in the presence of 0.5 mL artificial saliva. The sublingual administration of the drug-loaded tablets in the rat produced a dose-related increase in bladder contraction pressure.

Keywords

Heptapeptide; oral disintegrating tablet; spinal cord injury; lyophilization; neurogenic bladder dysfunction; neurokinin 2 agonist

1. Introduction

Neurogenic bladder dysfunction is a disorder arising from damage to the central nervous system that results in a lack of control of bladder control manifesting as either incontinence or urine retention (Jamison et al., 2013). In the United States, prevalence of bladder dysfunction is especially high among patients with spinal cord injuries (70–84%), Parkinson's disease (37–72%), and multiple sclerosis (70–84%) (Dorsher and McIntosh, 2012; Manack et al., 2011). In individuals with urinary retention, intermittent catheterization is recommended as a first-line option for bladder emptying (Hunter et al., 2013; Pannek et al., 2013). However, long-term catheterization presents its own set of problems, including recurrent urinary tract infections, urethral trauma, and increased hospital admissions (Stöhrer et al., 2009; Wyndaele, 2002). These secondary events ultimately incur significant costs for both the individual and the healthcare system (Chancellor et al., 2006; Yilmaz et al., 2014; Singh et al., 2011). Development of a pharmaceutical product to facilitate rapid and convenient voiding would be of great benefit to individuals with urinary retention: reducing adverse events, and improving quality-of life. Neurokinin A, a neurotransmitter released from neurons by activation of G-protein-linked receptors, acts on neurokinin-2 (NK₂) receptors to produce smooth muscle contractions in the bladder (Patak and Story, 2000; Yoshimura and Chancellor, 2003). NK₂ receptors are expressed at peripheral sites in the micturition pathway; targeting these receptors provides a promising therapeutic approach

for the treatment of bladder dysfunction in individuals with neurologic conditions (Palea et al., 1996; Schmidt et al., 2003).

[Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10) (LMN-NKA), was introduced over two decades ago by Chassaing and is an 803 Da heptapeptide which acts as a selective NK₂ receptor agonist that displays a 9 fold increase in functional and binding potency at the human NK₂ receptor compared to neurokinin A (4–10) (Chassaing et al., 1991; Warner et al., 2001; Patak and Story, 2000). Multiple *in vitro* and *in vivo* studies have confirmed the role of NK₂ receptors in inducing smooth muscle contraction of the bladder and gastrointestinal (GI) tract (Burcher et al. 2000; Warner et al. 2001; Sellers et al. 2006; Tramontana et al. 1998). In a recent publication, our research group demonstrated that intravenous (i.v.) administration of LMN-NKA to anesthetized acute spinal cord injured rats produces a direct smooth muscle contraction of the bladder and subsequent bladder emptying (Kullmann et al., 2017). However, i.v. administration as an ‘on demand’ multi-use longterm therapy is not practical. Oral administration of therapeutic agents for GI absorption is the most common and convenient delivery method for chronic therapy, but peptides, like LMN-NKA, are susceptible to hydrolysis and enzymatic degradation in the GI tract following oral administration (Di, 2015). In order to protect the integrity of orally administered peptides, alternative formulations and delivery routes must be developed to ensure efficacy of these therapies.

Oral transmucosal administration of peptide drugs offers an advantage over traditional oral delivery as this mode of delivery circumvents first-pass hepatic metabolism and prevents degradation in the GI tract. Several formulations and strategies have been employed for transmucosal peptide delivery (Aguirre et al., 2016; Morales et al., 2017). Among these, oral disintegrating tablets (ODTs) have gained attention as an emerging strategy to promote compliance and offer a convenient mode of administration (Montgomery et al., 2012). An ODT is a solid dosage form that disintegrates rapidly, typically within 30 sec, in the buccal cavity (FDA, accessed Aug. 20, 2009.). These features make ODTs an attractive choice for peptide delivery of a short acting, ‘on demand’ therapy for voiding. Current strategies for manufacturing ODTs include lyophilization, spray drying, wet granulation, and direct compression. Lyophilization is known to be useful particularly for heat sensitive biologics and can be used to manufacture ODTs with high porosity and rapid disintegration time (Sznitowska et al., 2004; Saharan, 2017); the first FDA-approved ODT in the United States was the Zydys[®] Claritin Reditabs (Schering Plough, Kenilworth, NJ. 1996) manufactured through the lyophilization process (McLaughlin et al., 2009).

The present study describes a formulation strategy for lyophilized, rapidly disintegrating LMN-NKA-containing ODTs for rapid acting, on-demand voiding therapy. To our knowledge, this is the first study reporting the LMN-NKA delivery in the solid oral dosage form for the treatment of neurogenic bladder dysfunction. A multivariate statistical design of experiment was applied to design ODT formulations with varying ratios of gelatin, glycine, and sorbitol as excipients. These formulations were modified to produce ODTs with rapid disintegration times and appropriate hardness. The final tablet formulation was evaluated for disintegration time, *in vitro* dissolution, stability, and ultimately for *in vivo* efficacy in

anesthetized, acutely spinalized female rats under isovolumetric bladder pressure recording conditions.

2. Materials and methods

2.1. Materials

Gelatin type B and glycine (ACS agent, 98.5%) were purchased from Fisher Scientific (Pittsburg, PA, USA). Sorbitol Solution USP (70%) was obtained from Spectrum Chemical MFG Corp (Gardena, CA, USA). HPLC grade trifluoroacetic acid and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). LMN-NKA was synthesized for Dignify Therapeutics (Durham, NC, USA) by Bachem America Inc (Torrance, CA, USA). GR159897 (NK₂ receptor antagonist; Tocris, Bristol, United Kingdom) was solubilized at 50 mM in DMSO and diluted with saline to a concentration of 1 mg/mL.

2.2. HPLC analysis

All samples were analyzed using the Shimadzu-HPLC gradient system (LC-20AD system, Shimadzu Corporation, Kyoto, Japan) equipped with a YMC-Pack Pro C18 chromatography column (150 × 4.6 mm, 3 μm, YMC America, Inc., Allentown, PA, USA) and an auto-sampler (SIL-20 HT, Shimadzu Corporation, Kyoto, Japan). The mobile phase was composed of double deionized (DDI) water containing 0.1% trifluoroacetic acid (A) and HPLC grade acetonitrile containing 0.1% trifluoroacetic acid (B) at a flow rate of 0.9 mL/min. Samples were kept at 4 °C until analyzed. After the injection of a 10 μL sample, separation was performed at 45 °C using a column temperature controller (CTO-20A, Shimadzu Corporation, Kyoto, Japan) by an A:B gradient from 95:5 to 90:10 v/v over 5 min, then to 85:15 v/v over 3 min, and to 65:35 v/v over 20 min. The gradient remained at 65:35 v/v for an additional 2 min before the system was re-equilibrated at 95:5 v/v for 5 min. The total time was 35 min. LMN-NKA was detected at a retention time of 22.43 min using a photodiode array detector (SPD-M20A, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 220 nm. The amount of LMN-NKA was calculated using a calibration curve obtained from the known LMN-NKA concentrations in the range of 10 to 500 μg/mL in water. Before analysis, all samples were diluted with water to fit into the calibrated concentration range.

2.3. Physicochemical Characterization of LMN-NKA

2.3.1. Ionization Constant (pK_a)—LMN-NKA was dissolved in deionized water at a concentration of 1 mM. The LMN-NKA solution was then titrated with 0.1 N HCl, followed by 0.1 NaOH and each treatment was repeated in triplicate. After each addition, pH values were measured by a S20 SevenEasy™ pH meter (Ag/AgCl reference) and glass electrode (Mettler Toledo, Columbus, OH, USA).

2.3.2. Partition Coefficient Determination (Lipophilicity)—The pH-dependent partition coefficients were calculated by measuring the partition of LMN-NKA between 1-octanol and aqueous buffers. Phosphate (pH 3), acetate (pH 4 and 5), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7), and tris(hydroxymethyl)aminomethane (TRIS, pH 8) buffer solutions were prepared at 100 mM concentrations (I=0.1, NaCl). Each

buffer solution was mixed with 1-octanol at a volume ratio of 1:15 (buffer:1-octanol) and pre-saturated in a shaker overnight. Each pre-saturated buffer solution (12.5 mL) was added to 1 mL of LMN-NKA buffer solution (0.15 mg/mL) and the resultant mixture was kept at 25 °C for 24 hours. An aqueous sample (200 μ L) obtained through centrifugation (5000 rpm, 10 min) was injected into the Shimadzu-HPLC gradient system (LC-20AD system, Shimadzu Corporation, Kyoto, Japan) to analyze the concentration of LMN-NKA in the aqueous phase. To analyze the concentration of drug in the initial LMN-NKA solution, 1 mL (0.15 mg/mL) was diluted with the HPLC mobile phase (12.5 mL) and a 200 μ L sample was withdrawn for HPLC analysis. A concentration of drug in the octanol phase was then calculated by subtracting the LMN-NKA concentration in the aqueous phase from the initial buffer concentration.

2.4. Experimental Design

A multivariate statistical design of experiment (DOE: The Design-Expert software program, version 8, Stat-Ease Inc., Minneapolis, MC) was applied to design ODT formulations with varying amounts of gelatin (X_1), glycine (X_2), and sorbitol (X_3). Chosen responses were the tablet disintegration time (Y_1) and structural integrity (Y_2). All analyses were performed using JMP® 12.0.1 software (SAS institute Inc., Cary, NC, USA).

2.5. Preparation of ODTs

The LMN-NKA ODTs were prepared by lyophilization method. Stock solutions of each excipient were prepared in DDI water. Gelatin was dissolved in DDI water at a concentration of 15% w/v with constant stirring at 40 °C. An aqueous solution of glycine at a concentration of 30% w/v was prepared in DDI water. The 30% sorbitol solution was prepared by diluting a 70% solution of sorbitol in DDI water. These three excipient solutions were combined in different ratios and vortexed for uniform mixing. The LMN-NKA stock solution (4% w/v in DDI water) was added to each mixture at room temperature (RT) to prepare ODT formulations containing either 1.5 mg or 5 mg of LMN-NKA. Aliquots (200 μ L) of each LMN-NKA ODT formulation solution were then transferred into plastic molds (9 $\times$ 4 mm), frozen at –80°C for 30 min and lyophilized under vacuum (100 mTorr) by a step gradient method using a VirTis Advantage benchtop tray lyophilizer (The VirTis Company, Inc., Gardiner, NY, USA) with VirTis AdVantage Wizard 2.0 (The VirTis Company, Inc., Gardiner, NY, USA) (Fig. 1). Prior to use, the prepared LMN-NKA ODTs were kept at room temperature in a nitrogen purged container with desiccant.

2.6. Characterization of blank and LMN-NKA-loaded ODTs

2.6.1. *In vitro* Disintegration Test—Each ODT was placed into 2 mL of DDI water (37 °C) in a 10 mL glass test tube (1.5 cm diameter) and stirred continuously at 100 rpm using a magnetic stirring bar (10 mm $\times$ 3 mm). Disintegration time was defined as the time required for complete disintegration of each tablet where no solid residue remained. A digital stopwatch with 1 sec precision (Traceable®, VWR International, West Chester, PA, USA) was used to record the disintegration time.

2.6.2. Morphological Characterizations—Morphological characteristics including shape, color, and structural integrity of tablets were visually evaluated. Structural integrity,

including a handling test and adhesion property of the tablet to the plastic mold, was investigated via ease of transfer from the mold to High-Density Polyethylene (HDPE) bottles (VWR® High-Density Polyethylene Wide Mouth Bottle, VWR International, West Chester, PA, USA). Each lyophilized ODT was pushed out from the plastic mold and transferred to a HDPE bottle 20 cm away using blunt-nosed thumb forceps. Structural integrity was assessed qualitatively on a numerical scale from 1 (very fragile, transferable with breakage of tablets) to 5 (hard, transferable without damaging tablets).

2.7. Stability of ODTs

ODTs containing 1.5 mg and 5 mg LMN-NKA were placed in desiccated, nitrogen-purged HDPE bottles and stored under both ambient (25 °C/60% relative humidity (RH)) and accelerated (40 °C/75% RH) storage conditions. The stability of LMN-NKA-loaded ODTs was evaluated at 0, 15, 30, and 90 days by HPLC analysis. Tablets (N=3) were withdrawn from the bottles at each interval and were dissolved in 10 mL of DDI water prior to analysis using the established HPLC method. The amount of drug in each tablet was calculated from the calibration curve obtained with known LMN-NKA concentrations in the range of 10 to 500 µg/mL in DDI water.

2.8. In vitro dissolution of ODTs

As there are currently no regulatory (USP or FDA) guidelines available for ODT dissolution tests, an *in vitro* dissolution test was performed using a modified dissolution method. The test was conducted by adding either fresh (<1 week) or stored (5 months, RT) ODTs containing 1.5 mg or 5 mg of LMN-NKA to a 20 mL beaker with 10 mL of phosphate buffered saline (pH 6.8) followed by continuous stirring at 50 rpm using a 6 × 25 mm magnetic stir bar (VWR International, West Chester, PA, USA) (Marques et al., 2011). At predetermined time intervals (0.25, 0.5, 1, 2, 5 and 15 min), 150 µL samples were withdrawn from the media and immediately replaced with an equal volume of fresh dissolution medium to maintain constant volume. The amount of LMN-NKA in the collected samples was analyzed by HPLC.

2.9. In vivo efficacy of ODTs

2.9.1. Animals—Adult female Sprague-Dawley rats (230–300 g, N=17, Charles River Laboratories, Inc., Raleigh, NC, USA) were housed under standard laboratory conditions with *ad libitum* access to water and food. All experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Integrated Laboratory Systems animal care and use committee. All efforts were made to minimize animal stress and to reduce the number of animals used.

2.9.2. Surgical Procedures—Rats were anesthetized with urethane (1.2–1.4 g/kg subcutaneous) and body temperature was maintained at 37 °C by a heated blanket. The carotid artery and jugular vein were cannulated to allow for blood pressure measurement and i.v. drug delivery, respectively. The abdomen was opened and the ureters were ligated and distally cut. Saline-filled polyethylene tubing with a flared tip (PE 50) was inserted through a small incision in the dome of the bladder and tied in place. The bladder cannula was connected to a T-tube for saline infusion (PHD2000 infusion pump, Harvard Apparatus,

Holliston, MA, USA) and bladder pressure measurement (Deltran®II Disposable Pressure Transducer, Utah Medical Products Inc, Midvale, UT, USA). The bladder pressure signal was amplified using a bridge amplifier (Transbridge 4M, World Precision Instruments, Sarasota, FL, USA). The rats were subjected to a complete spinal cord transection at the T9–T10 level following exposure of the vertebrae and a laminectomy (Tanahashi et al., 2012). This acute spinal cord injury (SCI) model served to mimic the ‘areflexic bladder’ which follows spinal injury.

2.9.3. Isovolumetric Cystometry—Functional bladder capacity was determined and the bladder was prepared for isovolumetric cystometry by the method previously described (Kullmann et al., 2017). Once bladder capacity was determined, the external urethra was clamped and the bladder filled to 70% of functional bladder capacity, where it remained for the duration of the experiment. Each rat was given saline vehicle and then an intravenous dose of LMN-NKA (1–10 µg/kg). Animal were subsequently given an ODT containing varying doses of LMN-NKA (0–5 mg/ODT). The rat oral cavity was swabbed to clear any debris prior to placing an ODT under the tongue and 50–200 µL of water was added to facilitate tablet disintegration. To allow bladder to return to stable pressure following i.v. administration, the ODTs were administered 30–60 minutes after the i.v. dose was given.

2.9.4. Parameters Measured—Maximal bladder pressure, bladder pressure response (maximal bladder pressure minus pre-treatment baseline pressure), time from drug administration to peak bladder pressure (time to peak), and duration of action (time from drug administration until bladder pressure returned to within 5 mmHg of pre-treatment bladder pressure) were recorded.

2.9.5. Data Analysis and Statistics—Data were recorded using LabChart software 7.0 (ADInstruments Ltd., Colorado Springs, CO, USA) and analyzed using both Excel (Microsoft, Redmond, WA, USA) and Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean and standard deviation of the mean.

3. Results and discussion

3.1. Physicochemical Characteristics of LMN-NKA

Protonation constants for LMN-NKA were determined by potentiometric titration using the Bjerrum graphical method (Kraft, 2003), a method commonly used to estimate pK_a values of polyprotic acids. Potentiometric titration data (Fig. 2A) were analyzed by the Niel Bjerrum Graphical method for a three- pK_a system as shown in Eq.1, where $\bar{n}_H$ is an average number ligand-bound dissociable protons (Kraft, 2003)

$$\bar{n}_H = \frac{K_{a1}[H^+] + 2K_{a1}K_{a2}[H^+]^2 + 3K_{a1}K_{a2}K_{a3}[H^+]^3}{1 + K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2 + K_{a1}K_{a2}K_{a3}[H^+]^3} \quad \text{Eq. 1}$$

The $\bar{n}_H$ calculated from the experimental result was plotted against the corresponding pH value (Fig. 2B) to produce the Bjerrum plot. The values estimated from the Bjerrum plot were used as seed values for refinement in HyperQuad suite program (Gans et al., 1996;

Ferdousiet al., 2016) to yield the actual pK_a values. The values were recorded to be 11.38 ± 0.08 , 7.71 ± 0.07 and 4.73 ± 0.15 as shown in Fig. 2C. Visualization of net charge and $\log P$ values for LMN-NKA across the pH range 3–8 are shown in Fig. 3. As the pH increases from 4, the net charge of LMN-NKA is predicted to decrease. The fraction of LMN-NKA in the octanol phase was higher at pH values greater than 4 which suggests that the absorption of LMN-NKA increases as charge decreases. The pH values of the final LMN-NKA ODT formulation in water and saliva were found to be 5.8 and 6.4, respectively by an Orion Star™ A111 pH meter (Thermo Fisher Scientific, Waltham, MA, USA). No further modification of ODT formulation was undertaken based on the pH values since it was intended to develop LMN-NKA ODT using the simplest formulation to test for proof-of-concept efficacy.

3.2. LMN-NKA ODT Formulation

Formulation of a lyophilized ODT with acceptable mechanical properties and expedient drug release requires precise selection of appropriate excipients. For this formulation, highly water soluble compounds such as gelatin, glycine, and sorbitol were selected. Gelatin serves to form a supporting matrix- its ability to create a glassy and amorphous structure which provides the tablet with structural strength for easy handling (Seager, 1998; Sudhakar et al., 2006). Despite this benefit, high concentrations of gelatin can increase disintegration time (Gugulothu et al, 2015). Therefore, optimizing the gelatin concentration was critical to obtain the ideal ODTs with sufficient strength and a conserved disintegration rate. Sorbitol was chosen as a filler material due to its high water solubility and pleasant taste. Additionally, sorbitol acts to protect the lyophilized tablet from structural collapse or shrinkage during the freeze drying process (Goel et al., 2008). Glycine was incorporated into the formulation in an effort to accelerate disintegration time. Fukami *et al.* demonstrated that amino acids such as glycine, L-tyrosine, and L-alanine have large polar surface free energies with a strong affinity to water. These characteristics enhance wetting and disintegration time (Fukami et al., 2005; Matsuoka et al., 2007). Moreover, glycine has previously been applied to enhance the stability of protein and peptide formulations (Challener, 2015; Passot and Tréleá, 2016).

DOE was employed to semi-optimize ODT formulations by evaluating the individual and combined effects of the formulations variables. A $3^{1.2^2}$ full factorial design with 12 test runs generated by JMP software was carried out. The results in Table 1 show that the tablet disintegration time varied from 1 to 15 sec. Tablet structural integrity, including handling test and adhesion, which was assessed qualitatively, ranged from 1 (very fragile, transferable with breakage of tablets) to 5 (hard, transferable without damaging tablets). Results from 12 test runs were used to estimate the significance of the model using ANOVA testing. A model was considered significant if the p value was less than 0.05 at a 95% confidence level which suggests that the variability of each factor is within the acceptable range for optimizing expected response. The DOE results showed that the amount of gelatin had a significant effect on disintegration time (Y_1 , $p = 0.0056$) and tablet structural integrity (Y_2 , $p = 0.0033$). Higher gelatin concentration allow for a greater crosslinks between gelatin fibers; as such, higher proportions of gelatin generated harder tablets with increased disintegration times (Djagny et al., 2001). Similar behavior was reported by Shoukri et al. (Shoukri et al., 2009).

The working ODT formulation was determined to be 3% (w/v) gelatin, 2% (w/v) glycine, and 1% (w/v) sorbitol in DDI water approximated from the theoretically predicted values from the 12 experimental runs. Further optimization is planned to using a mixed experimental design to examine the surface response map, after sufficient *in vivo* studies have been performed to confirm that lyophilized ODTs are the delivery method of choice for development. Using this formulation, blank and drug-loaded ODTs with 1.5 mg and 5 mg of LMN-NKA were prepared for further studies.

3.3. ODT specifications

The prepared ODT was microscopically porous in the form of a circular cylinder with hemispheres attached at both ends ($9.7 \times 5.9 \times 3.9$ mm, length $\times$ width $\times$ height). The weight of each tablet was recorded and ranged from 10.33 to 10.62 mg. There was no significant change in firmness when LMN-NKA was added, but the disintegration times increased from ~20 to ~30 sec. This change is likely attributed to decreased porosity of the tablet due to a smaller amount of water compared to the blank matrix. The content of LMN-NKA in the final tablet formulation was $106.4 \pm 2.0\%$ of label strength, which is within 85–115% of the target dose with a relative standard deviation (RSD) less than or equal to 6% (United States Pharmacopeial Convention, 1999). The LMN-NKA content in a tablet divided into fourths was between 24.60% and 25.12% of the total drug content, indicating that the drug was uniformly distributed.

3.4. ODT Stability

Stability of LMN-NKA in the optimized formulation was evaluated under two storage conditions (25 °C/60% RH and 40 °C/75% RH) as per the International Council for Harmonisation (ICH) Guidelines on the stability testing of New Drug Substances and Products Q1A(R2)(FDA, 2003; Guideline, 2003). There were no significant changes in appearance of the tablets after 90 days of storage under either condition. Drug content, 1.5 mg and 5 mg of LMN-NKA per tablet, and stability over 90 days were confirmed by HPLC analysis. Prior to conducting the HPLC assay for LMN-NKA ODTs, it was confirmed that the blank ODT did not produce any interfering peaks from the excipients at the retention time of LMN-NKA. The results from stability studies after 14, 28 and 90 days storage are presented in Table 2. Drug content in the 5.0 mg LMN-NKA ODTs after 14, 28 and 90 days of storage at 25 °C/60% RH were 5.03 ± 0.06 , 5.23 ± 0.05 , and 5.11 ± 0.02 mg, respectively. Drug content of tablets stored at 40 °C/75% RH, evaluated at the same time points, was 4.96 ± 0.06 , 4.82 ± 0.23 , and 4.92 ± 4.92 mg, respectively. Results indicate that the 5.0 mg LMN-NKA tablet is stable for 90 days under to the ICH guidelines Q1A on the stability of drug products. The lower dose, 1.5 mg tablet, was also stable for 90 days under the same storage conditions (Table 2).

3.5. In vitro dissolution of ODTs

To ensure the complete release of LMN-NKA from ODTs, an *in vitro* dissolution test was performed using phosphate buffered saline solution adjusted to pH 6.8 to simulate saliva. This buffered solution was previously used as an *in vitro* dissolution method for testing the dissolution of acyclovir (FDA, 2015). The dissolution profiles of LMN-NKA from ODTs stored for 0 days and 5 months are presented in Fig. 4. Cumulative percentage of LMN-

NKA released from each ODT was more than 97% after a 15-min incubation period at 37 °C. These results indicate a complete release of drug within 15 min. The dissolution rate and extent of drug release from fresh and stored ODTs did not differ over the dissolution period tested. The amount of drug released over 15 sec from the 1.5 mg and 5 mg ODTs ranged from 72.26% to 79.50% and from 54.99% to 67.79%, respectively. The results indicate that lower dose ODTs have a higher initial release of drug. Based on the results from *in vitro* dissolution tests and disintegration studies, the LMN-NKA ODTs can release drug within the target 30 sec time interval.

3.6. In vivo studies

To determine the efficacy of LMN-NKA ODTs, the anesthetized acute SCI female rats were given increasing doses of LMN-NKA. Under isovolumetric conditions with the bladder filled to 70% capacity, sublingual placement of ODTs containing 0.1 – 5 mg of LMN-NKA produced a dose-dependent increase in bladder pressure (Fig. 5A and 5B). The time to reach peak bladder pressure was slower with ODT (5–8 min) compared to i.v. (0.5 min) drug administration (Fig. 5C). This was expected as it takes additional time for the drug to be absorbed through the oral mucosa. The LMN-NKA ODT mediated increase in bladder pressure was readily reversed with application of the NK₂ receptor antagonist GR 159898 (1 mg/kg i.v.) (Fig. 5D). This supports the hypothesis that bladder response was mediated by NK₂ receptors. Application of blank ODTs without any LMN-NKA did not change bladder pressure (data not shown).

The anesthetized, acute spinal injured rat is a suitable and well accepted model for the study of neurologically induced underactive bladder and is widely used to explore the action of novel urologic compounds on the bladder (Lee et al., 2014; Shi et al., 2013). The rat is less ideal for examining the efficacy of ODTs due to a thick keratinized layer of the rat oral mucosa which impedes transmucosal absorption (Harris and Robinson, 1992; Rathbone et al., 2002). Nevertheless, the current data demonstrate that administration of ODTs containing LMN-NKA can initiate a bladder contraction. With this model, feasibility of an ODT delivery system was confirmed. Future strategies are needed, such as absorption enhancers or buccal film delivery or intranasal delivery in order to fully evaluate the optimal transmucosal delivery of LMN-NKA and to ensure the safety profile in humans.

4. Conclusions

A heptapeptide, [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10) was successfully formulated as an oral disintegrating tablet using a lyophilization technique. The dissolution and disintegration profiles of the prepared ODTs followed the compendial FDA regulations. LMN-NKA administered sublingually via an ODT showed good efficacy in an *in vivo* SCI rat model. While the ODTs were able to match the efficacy of the i.v. administration, the potency of ODTs was orders of magnitude lower compared to i.v. administration. This slower onset and lower potency of ODTs is in part due to poor absorption through the rat's heavily keratinized oral mucosa. Despite these promising results, it is important to continue to seek alternative formulation strategies for transmucosal absorption of LMN-NKA, such as buccal film or intranasal delivery, or utilization of absorption enhancers. ODTs containing LMN-NKA are

a promising dosage form for on-demand voiding therapy and offer a stable and effective oral therapy with a rapid onset of action. Formulations like LMN-NKA ODTs could provide an attractive alternative for patients suffering from neurogenic bladder dysfunction and could replace or supplement urinary catheter use as they can be self-administered and effectively to induce bladder contractions and facilitate micturition.

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Figure 1. Lyophilization protocol of LMN-NKA ODTs; chamber pressure (dashed line) and shelf temperature (solid line)

Figure 2.
 (A) Potentiometric titration curve for LMN-NKA with volume of 1 M KOH added, (B) Bjerrum difference plot of n_{H}^- versus pH for LMN-NKA, and (C) molecular structure of LMN-NKA with pK_a values of ionizable side chains.

Figure 3. pH-dependent net charge distribution performed by regression analysis of titration curves using the HyperQuad program (red solid line) and log P values of LMN-NKA at pH 3, 4, 5, 7, and 8 (blue circle).

Figure 4. Dissolution profile for ODTs containing 1.5 (●, ○) and 5 mg (▲, △)LMN-NKA. Fresh ODTs made within 1 week of testing (solid line) and 5 month ODTs stored at RT (dashed line) were tested in phosphate buffered saline (pH 6.8) at 37 ± 0.5 °C. Data are shown as mean $\pm$ SD (N=3).

Figure 5.

ODTs containing LMN-NKA produce a rapid dose-related NK₂-mediated increase in bladder pressure. (A) Physiograph tracings showing ODT-LMN-NKA induced increase in bladder pressure with increasing doses (0.7–3.6 mg/kg, dose given at each arrow). (B) Bar graph shows LMN-NKA ODT mediated increase in bladder pressure, compared to 3 µg/kg i.v. LMN-NKA. Bars represent the mean + SD of the bladder pressure response above baseline. Baseline pressures were 8–15 mm Hg. N=5–12 for each dose group. (C) Physiograph trace comparing i.v. and ODT administration of LMN-NKA. Doses given at arrows. [D] Physiograph trace illustrates reversal of the ODT LMN-NKA (4.6 mg/kg) effect by the NK₂ receptor antagonist GR159898 (1 mg/kg i.v.). Doses given at arrows.

Table 1

A full factorial design with 12 experimental runs generated by JMP software and characterization data.

Run	Factors			Responses		
	X ₁ : Amount of gelatin (mg)	X ₂ : Amount of glycine (mg)	X ₃ : Amount of sorbitol (mg)	Y ₁ : <i>In vitro</i> disintegration time (s)	Y ₂ : Structural integrity	
					Handling test* (1-4)	Adhesion**
F1	20	10	10	1	2	—
F2	20	10	20	6	2	—
F3	20	20	10	1	2	—
F4	20	20	10	1	2	—
F5	40	10	10	4	3	+
F6	40	10	20	5	2	—
F7	40	20	10	3	2	+
F8	40	20	20	3	2	—
F9	80	10	10	7	4	+
F10	80	10	20	15	4	+
F11	80	20	10	10	4	+
F12	80	20	20	6	3	+

* 1 very fragile, 2 fragile, 3 durable, 4 hard

** +transferable from the plastic mold to HDPE bottles without damaging the tablets, -transferable with breakage of the tablets

Stability of LMN-NKA (1.5 mg and 5 mg) in ODT under long-term storage conditions (25 °C/60% relative humidity) and under accelerated conditions (40 °C/75% relative humidity) for up to 90 days. Data shown as mean $\pm$ SD (N=3).

Table 2

Condition (°C/%RH)	Claimed Strength (mg)	Day 14		Day 28		Day 90	
		Actual Strength (mg)	% Label Claim	Actual Strength (mg)	% Label Claim	Actual Strength (mg)	% Label Claim
25/60	1.5	1.59 $\pm$ 0.03	106.4 $\pm$ 2.0	1.56 $\pm$ 0.01	103.8 $\pm$ 0.8	1.46 $\pm$ 0.02	97.2 $\pm$ 1.6
25/60	5	5.03 $\pm$ 0.06	100.7 $\pm$ 1.3	5.23 $\pm$ 0.05	104.7 $\pm$ 1.0	5.11 $\pm$ 0.02	102.2 $\pm$ 0.3
45/75	1.5	1.59 $\pm$ 0.08	105.7 $\pm$ 5.5	1.59 $\pm$ 0.08	104.6 $\pm$ 0.7	1.46 $\pm$ 0.02	97.4 $\pm$ 1.1
45/75	5	4.96 $\pm$ 0.06	99.1 $\pm$ 1.2	4.82 $\pm$ 0.23	96.5 $\pm$ 4.5	4.92 $\pm$ 0.11	98.4 $\pm$ 2.3